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**13. ABSTRACT** *(Maximum 200 Words)*

Metastasis to bone is a common and serious complication of breast cancer. The aim of this project is to use our unique mouse model of breast cancer metastasis to bone to identify the molecular basis for this problem. In the first year, we have investigated the expression of a panel of candidate genes in tumor cells that metastasize to bone compared to those that do not metastasize or that metastasize to other organs. Many of these candidate genes are expressed in the tumor cells and some may be differentially expressed. We have also measured expression of chemokine receptors in these tumor cells but have yet to find one(s) that correlate with bone specific metastasis. To determine the role of osteolysis in metastasis to bone, inhibitors of bone resorption such as osteoprotegerin (OPG) have been investigated. Treatment of tumor bearing mice with OPG appears to reduce metastatic tumor growth in bone and overexpression of OPG reduces tumor cell growth, suggesting that osteolysis is important in the growth regulation of tumor cells. To investigate the role of stromal derived factors in metastasis to bone, beta3 integrin, M-CSF, MMP9 and MMP2 knockout mice have been bred onto the Balb/c background.

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## INTRODUCTION
Breast cancer is the largest cause of cancer related deaths in western women. Bone metastases are found in around two thirds of patients with metastatic disease and are a major factor in the morbidity and mortality associated with breast cancer. The vast majority of metastatic breast cancers cause osteolytic lesions that result in severe pain, pathological fractures, spinal cord compression and hypercalcemia. The basis for the proclivity of breast cancer cells to growth in bone and activation of osteoclasts is not well understood and identification of the factors responsible has been hampered by the lack of a suitable animal model.

We have recently developed a mouse model of breast cancer metastasis to bone that involves the entire metastatic process (Lelekakis *et al.*, 1999). In this model, tumor cells injected **orthotopically** into the mammary fat pad of syngeneic mice develop into a primary mammary carcinoma and **spontaneously metastasize** to various organs, including bone (Figure 1).

The aim of this project is to identify factors that are important in the establishment of bone metastases. **The specific aims of this project are:**
To determine the factors expressed by metastatic breast tumor cells and/or bone stromal cells in the bone microenvironment that regulate tumor cell growth and/or osteoclast development.
To investigate the extent of osteoclast involvement in tumor mediated osteolysis.
To investigate the mechanisms of osteoclast activation by breast tumor cells

## BODY
**Task 1. To determine the expression of cytokines and osteoclast regulatory factors by breast tumor and bone stromal cells (months 1-12)**
Expression of osteoclast regulatory factors and cytokines by tumor cells has been implicated in the progression of bone metastases. Our orthotopic mouse model enables us to investigate in detail the expression of a selection of candidate genes (see Table 1) in the metastatic tumor environment and to compare this expression with that in the primary tumor. The expression patterns of the selected molecules are to be examined by immunohistochemistry (IHC) and *in situ* hybridization (ISH). Construction of tumor arrays is underway, but has not yet been completed. The order in which some of the tasks for this project are being performed has therefore been adjusted, with parts of Tasks 2 and 3 brought forward so that all the originally planned tasks will be completed. However, we have analyzed tissue sections by IHC to determine the expression pattern of PTHrP. Both primary tumors and metastatic tumor deposits of the bone metastasizing 4T1.13 tumor line stain strongly for PTHrP, as do some bone cells (Figure 2). Interestingly, tumors that do not metastasize to bone also stain strongly for PTHrP when injected and grown intra-tibially. These data suggest that PTHrP expression does not correlate with bone metastasis in our model. This is an interesting finding given that the majority of experimental data supports a role for PTHrP expression in bone metastasis (Guise *et al.*, 1996, Thomas *et al*, 1999), whereas clinical data does not (Henderson *et al.*, 2001). Further analysis of the role of PTHrP is described under Task 3.

We have also examined the expression of the candidate genes by performing reverse transcriptase real time quantitative PCR (RT-RTQ PCR) on RNA extracted from primary tumors of the bone metastasizing line 4T1.2 and also on the lung metastatic (66cl4) and non-metastatic line (67NR) that comprise our unique model (Figure 3). This approach provides a simple assay

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of the differential gene expression profiles and will focus our attention on particular molecules within the originally selected group. The genes detected by RT-RTQ PCR include IL-1α, IL-6, uPA, MMP2, MMP9, RANKL and OPG. Surprisingly, since IHC has shown PTHrP to be expressed in primary tumors from the three tumor lines, minimal expression of PTHrP was detected. This assay will be repeated and additional primers for PTHrP (and other genes not detected) will be tested to validate this result. Since RT-RTQ PCR does not identify the cell types responsible for the expression and is difficult to perform on bone metastases, IHC and ISH will still be completed as proposed. Although we originally planned for this work to be completed within twelve months, it is now expected to be finalized within the next 6 months.

In addition to the molecules originally proposed, we have measured expression of a panel of chemokine receptors in the tumors. Chemokines have long been known to be important in the mobilization of immunological cells from thymus, spleen and bone marrow and for their ability to home to sites of infection or damage. A recent study (Muller *et al.*, 2001) highlighted the importance of chemokines in the homing of breast tumor cells to sites of metastasis, providing a possible mechanism whereby metastatic cells selectively colonize particular secondary organs depending on which chemokine receptor(s) they express. It was therefore of great interest to determine whether chemokine driven migration may be responsible for the different patterns of metastasis in our model. Expression of a panel of chemokine receptors was measured in the different primary tumors using RT-RTQ PCR (Figure 4). The chemokine receptors were found in varying levels in the tumors, with CCR1, CCR2, CCR5, CX3CR1 and CXCR1 showing the highest levels. However, expression of the ligands for these receptors is not confined to the sites to which the tumor cells metastasize (eg. bone and lung). This expression analysis has therefore not been able to elucidate if chemokine-chemokine receptor interactions are involved in organ specific metastasis in our model.

**Task 2. To investigate the extent of osteoclast requirement in tumor mediated osteolysis (months 1-18)**
Considerable evidence suggests that tumor cells cause bone destruction by recruiting osteoclasts (Taube *et al.*, 1994). The role of osteoclasts in our mouse model of breast tumor metastasis to bone is to be examined using osteoclast inhibitors and mice lacking functional osteoclasts. Osteoprotegerin (OPG) is a decoy receptor for the osteoclast differentiation factor, RANKL and therefore competitively inhibits osteoclast activation by osteoblasts and/or tumor cells (Yasuda et *al.*, 1999). One of the advantages of our model is that we have developed an RTQ-PCR assay to measure metastatic tumor burden in bone and other organs. Therefore, mice were injected with the bone metastasizing clone, 4T1.2, treated with daily injections of OPG using two different two-weekly regimens and metastatic tumor burden measured in bone and lungs (Figure 5). In both cases, the spines and femurs of mice treated with OPG displayed reduced metastatic tumor burden compared to controls. Since these results were not quite statistically significant, further experiments will be required to validate these results. Nevertheless, the data suggest that metastatic tumor growth in our model is at least partially dependent on osteolysis. We have also transfected tumor cells with an expression construct for OPG and experiments are underway to determine if OPG expressed by the tumor cells is more effective at inhibiting osteolysis and therefore metastatic tumor growth. An *in vivo* assay of metastatic tumor growth, involving injection of tumor cells directly into the tibia, was used to determine the effect of tumor cell OPG expression (Figure 6). Tumor cells overexpressing OPG showed reduced metastatic tumor

burden compared to control (vector transfected) tumor cells 14 days after tumor inoculation. These data provide encouraging indications that osteolysis is an important factor in the regulation of tumor cell growth in bone, probably via the release of stimulatory cytokines. Further experiments will help to strengthen these results and provide insights into the mechanism of this regulation.

Similar experiments to those described above are being performed with the bisphosphonate zoledronate and with the beta3 integrin peptidomimetic antagonist S247.

Mice lacking beta3 integrin or M-CSF (op/op), both of which display osteopetrotic phenotypes, are also being used to determine the requirement for osteoclasts. These mice have been backcrossed onto the Balb/c background that is required for tumor growth in our syngeneic model. The beta3 knockout mice are at generation F6 and the op/op mice are at generation F7. Further backcrossing will continue to produce generation F9 mice, which will then be suitable for tumor injection experiments. It is expected that this will be performed within the next 6 months.

**Task 3.  To examine mechanisms of osteoclast activation by tumor cells (months 12-36)**
The mechanism(s) by which tumor cells induce osteolysis are not well understood and the identification of factors important in this process is a major focus of this project. The role of OPG in osteolysis induced by metastatic tumor cells will be investigated as described in Specific Aim 2. In this section, we will investigate the role of other factors identified in Specific Aim 1 above to promote or inhibit osteolysis. It is expected that within the next 6 months we will have identified a number of these molecules and will be producing and/or procuring reagents to perform gain and loss of function experiments. In addition, the role of matrix metalloproteinases in our model is being examined by breeding MMP12 and MMP9 knockout mice onto the Balb/c background. These mice are at generation F8 and F9 respectively. The MMP9 mice have been tested for their ability to support 4T1.2 tumor growth and the colonies are currently being expanded for experimental purposes. The MMP12 mice will follow in approximately two months.

As mentioned above, there is considerable data suggesting that PTHrP is important in breast cancer metastasis to bone. We have investigated the role of PTHrP in our model of metastasis by genetically engineering altered PTHrP expression levels in tumor cell lines. Our data suggests that overexpression of PTHrP does not enhance the ability of tumor cells to metastasize to bone. However, inhibition of PTHrP expression using antisense cDNA constructs reduces the growth rate and therefore metastatic tumor burden of 4T1.2 tumor cells (Figure 7). These data imply that PTHrP may be required for tumor cell growth and that antisense PTHrP may block this intracellular function of PTHrP.
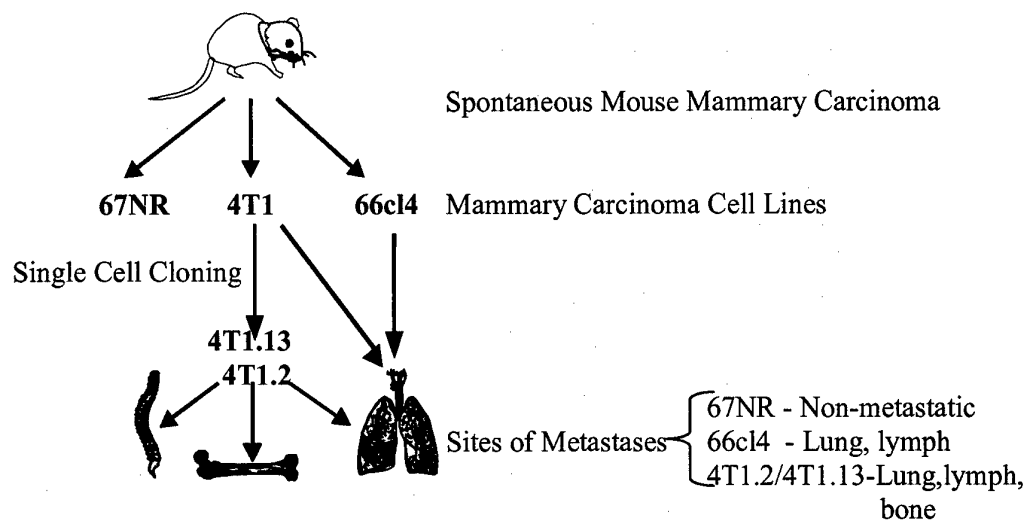
**TABLES AND FIGURES**



Spontaneous Mouse Mammary Carcinoma

67NR    4T1    66cl4    Mammary Carcinoma Cell Lines

Single Cell Cloning

4T1.13
4T1.2

Sites of Metastases
- 67NR - Non-metastatic
- 66cl4 - Lung, lymph
- 4T1.2/4T1.13-Lung,lymph, bone

**Figure 1. Mouse Model of Breast Cancer Metastasis to Bone**

**Table 1 – List of Candidate Genes**

| Gene | Function | Method/Availability of Reagents | | |
| --- | --- | --- | --- | --- |
| | | ISH[#] | IHC | Neutralizing Ab |
| IL-1 | Cytokine | 1 | Yes | Yes |
| IL-6 | Cytokine | 1 | Yes | Yes |
| IL-11 | Cytokine | 1 | Yes | Yes |
| IL-18 | Cytokine | 1 | Yes | Yes |
| TNFα | Cytokine | 1 | Yes | Yes |
| TGFβ | Cytokine | 1 | Yes | Yes |
| M-CSF | Cytokine | 1 | Yes | Yes |
| PTHrP | Hormone | 2 | Yes | Yes |
| RANKL | Osteoclast Differentiation | 2 | ? | ? |
| OPG | Decoy Receptor | 2 | ? | ? |
| RANK | RANKL Receptor | 2 | ? | ? |
| MMP2 | Protease | 2 | Yes | ? |
| MMP9 | Protease | 2 | Yes | ? |
| MT1-MMP | Protease | 1 | ? | ? |
| uPA | Protease | 2 | Yes | ? |

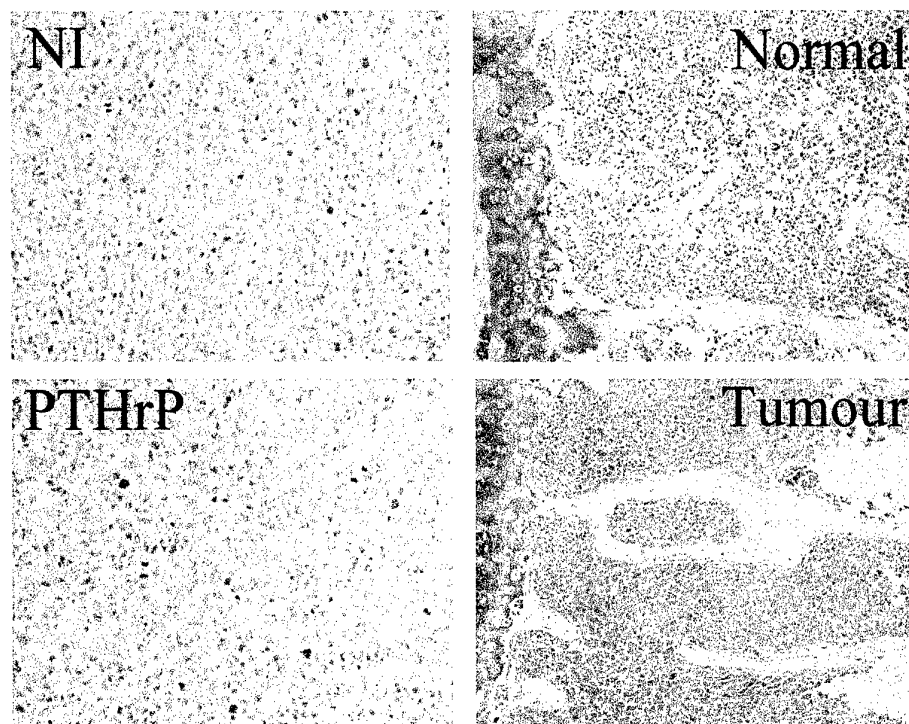[#] 1=cDNA to be PCR cloned , 2=cDNA in laboratory

**Figure 2. Immunohistochemistry Analysis of PTHrP Expression.** Sections were prepared from primary tumor and spine of mice bearing 4T1.13 tumor, stained with haematoxylin and eosin and immunostained with a peroxidase conjugated anti-synthetic human PTHrP antibody. Top Left: Section through primary tumor stained with pre-immune serum. Bottom Left: Section through primary tumor stained for PTHrP (brown staining). Top Right: section showing normal bone and bone marrow. Bottom Right: adjacent section showing large tumour deposit expressing PTHrP.
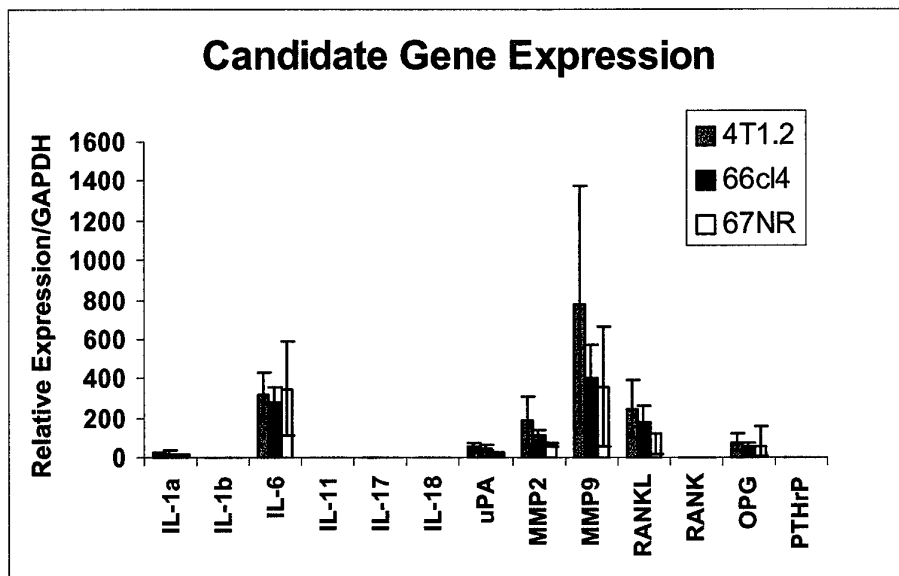
**Figure 3. Expression of Candidate Genes in Primary Tumors.** RNA was extracted from three primary tumors for each of the lines indicated. cDNA was synthesized and SyBr Green RT-RTQ PCR performed for each of the genes indicated. Expression relative to GAPDH was determined and error bars represent the standard error of the mean.
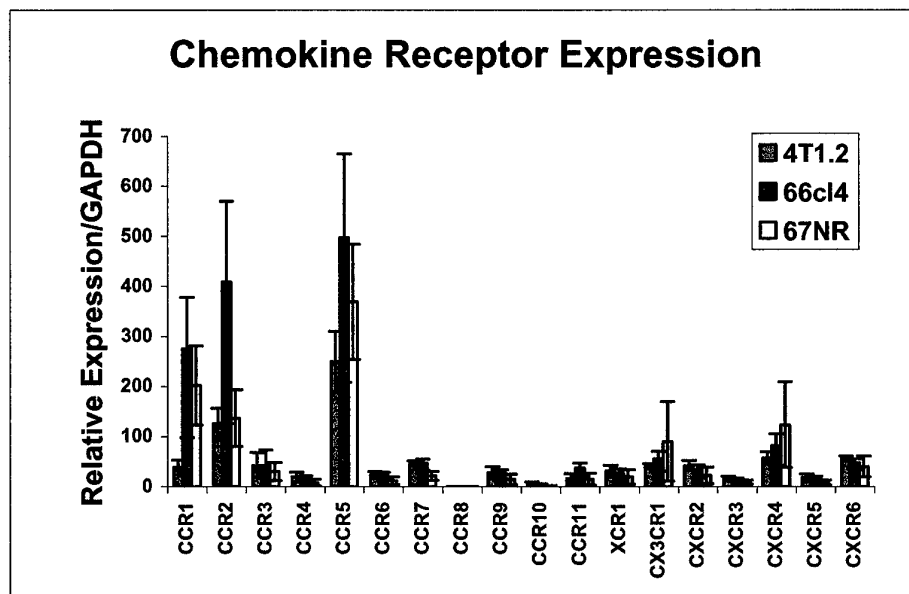


**Figure 4. Expression of Chemokine Receptors in Primary Tumors.** RNA was extracted from 3 separate primary tumors for each of the lines indicated. cDNA was synthesized and SyBr Green RT-RTQ PCR performed for each of the chemokine receptors. Expression relative to GAPDH was determined and error bars represent the standard error of the mean.
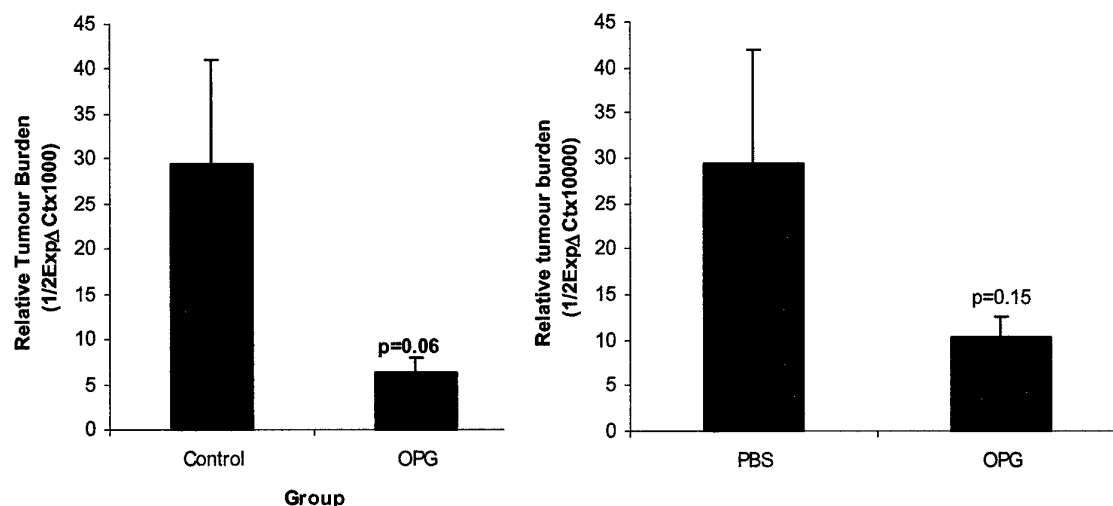
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**Figure 5. Effect of OPG on Metastatic Tumor Burden in Spine.** Mice were injected with a single cell clone of 4T1.13 cells transfected with pcDNA3 (4T1.13Con2) and primary tumors allowed to develop for either 11 days (Left Panel) or 14 days (Right Panel). OPG (1mg/kg/day) was then administered daily by subcutaneous injection for 14 days. Genomic DNA was prepared from spine and RTQ PCR used to determine metastatic tumor burden. Error bars represent standard errors of the mean and p values were determined using the student t test.
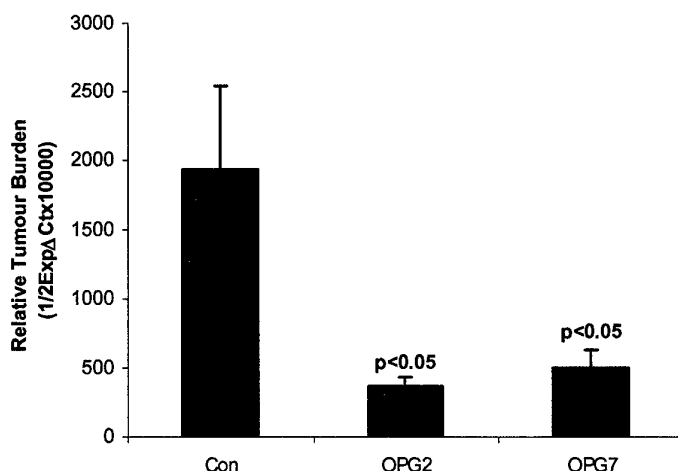


**Figure 6. *In Vivo* Growth of Tumor Cells Overexpressing OPG.** Two single cell clones overexpressing OPG and one vector transfected clone of 4T1.2 were injected directly into the mouse tibia and tumor cells allowed to grow for 14 days. Tibia were removed and tumor growth measured using RTQ PCR. Error bars represent standard error of the mean and p values were determined using the student t test.
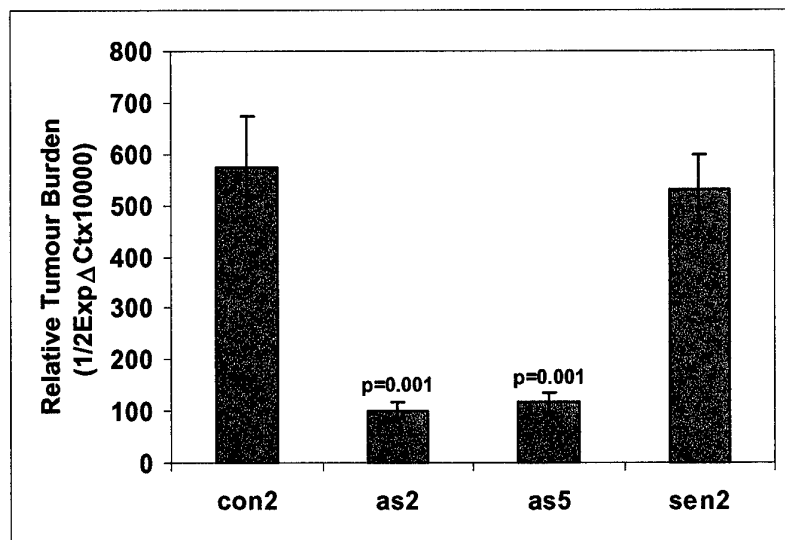
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**Figure 7. Effect of Antisense PTHrP on Metastatic Tumor Burden.** Single cell clones of 4T1.13 tumor cells were transfected with vector (con2), PTHrP sense (sen2) or PTHrP antisense (as2 and as5) and injected into the mouse mammary fat pad. Four weeks later, spines were removed and RTQ PCR used to measure metastatic tumor burden. Error bars represent standard error of the mean and p values were calculated using the student t test.

## KEY RESEARCH ACCOMPLISHMENTS

➢ Demonstration that PTHrP expression in primary tumors does not correlate with metastasis to bone

➢ Demonstration that increased PTHrP expression does not enhance metastasis to bone

➢ Demonstration that inhibition of PTHrP expression reduces tumor growth rate and subsequent bone metastases

➢ Profiling candidate gene expression in primary tumors by RT-RTQ PCR

➢ Profiling chemokine receptor expression in primary tumors

➢ Demonstration that administration of OPG reduces metastasis to bone and inhibits tumor cell growth in bone

➢ Back-crossing of mice lacking functional osteoclasts (beta3 integrin knockout, op/op) or deficient in MMP function (MMP9, MMP12 knockout) onto the Balb/c background

## REPORTABLE OUTCOMES

➢ Patent application for the use of RTQ PCR to measure metastasis of 4T1.13Con2 to secondary organs.

➢ Supervision of Joanna Kendrick who was awarded B.Sc. (Hons Class I) to for her work on the role of OPG in breast cancer metastasis in our mouse model

➢ Manuscripts in preparation:
Role of PTHrP in an orthotopic mouse model of breast cancer metastasis to bone. Tavaria, MD, Moseley, JM, Natoli, A, Lelekakis, M, Ho, P, Hards, D, Martin, TJ, Anderson, RL.

OPG inhibits metastasis in an orthotopic mouse model of breast cancer metastasis to bone. Tavaria, MD, Kendrick, J, Natoli, A, Lelekakis, M, Restall, T, Hards, D, Martin, TJ, Moseley, JM, Anderson, RL.

➢ Abstract and platform presentation at 4th International Conference of Cancer Induced Bone Diseases, Awaji Shima, Japan. Nov 2001. INHIBITION OF BREAST CANCER METASTASIS TO BONE IN AN ORTHOTOPIC MOUSE MODEL. (2001) Tavaria, MD, Natoli, A, Lelekakis, M, Ho, P, Hards, D, Martin, TJ, Moseley, JM, Anderson, RL.

➢ Abstract and poster presentation at 14th Lorne Cancer Conference, Victoria, Australia. Feb 2002. INHIBITION OF BONE METASTASES IN A MOUSE MODEL OF BREAST CANCER METASTASIS. (2002) Tavaria, MD, Natoli, A, Kendrick, J, Lelekakis, M, Restall, T, Sloan, E, Hards, D, Ho, P, Martin, TJ, Moseley, JM, Anderson, RL.

## CONCLUSIONS

In the first year of this grant, we have begun to characterize the expression of candidate genes. The expression profiles of these genes as determined by RT-RTQ PCR are described in Figure 3 and there is an indication that some of these genes may be differentially expressed. For example, expression of MMP9 is higher in 4T1.2 tumors than 66cl4 and 67NR, consistent with the greater invasiveness of 4T1.2 tumors. Expression of some candidate genes was not detected, but further experiments are required to confirm these results and to reduce the variation between assays. We have performed IHC and ISH on primary tumors and spine sections to determine PTHrP expression patterns. PTHrP is expressed in metastatic tumor deposits in the spine and interestingly, in all three primary tumors. Gain and loss of function experiments have been performed to investigate the role of PTHrP in metastasis to bone in our model. Overexpression of PTHrP in cells that do not normally metastasize to bone does not enhance bone metastasis, suggesting that other factors are more important in bone specific metastasis. Inhibition of PTHrP expression does however appear to reduce tumor cell growth and therefore indirectly affect metastatic tumor burden. These findings are interesting in light of the current debate on the role of PTHrP in breast cancer metastasis to bone and will be submitted for publication shortly.

Recent interest in the role of chemokine receptors in breast cancer metastasis led us to investigate their expression in our tumor cells. While many of the receptors are expressed by the tumor cells, their expression patterns have not yet enabled us to determine whether a specific chemokine-chemokine receptor pair is involved in organ specific metastasis in our model.

Most metastatic breast cancers that colonize bone cause increased osteolytic activity and therefore bone destruction. However, it is unclear whether tumor cells rely completely on osteoclasts or can also directly resorb bone. We are investigating the osteoclast dependence of tumor cells in our model using anti-osteoclastic factors such as OPG, bisphosphonates and anti-integrin peptidomimetics. Experiments so far indicate that OPG does inhibit the ability of tumor cells to grow in bone, thereby implying some dependence on osteoclastic bone resorption. Further experiments will examine the mechanisms of this inhibition and the effects of the other osteoclast inhibitors.

To investigate the role of stromal cell-derived beta3 integrin, MMP12, MMP9 and M-CSF, mice lacking these genes are being bred on to the Balb/c background for use in our tumor model. With the back-crossing almost complete, we will soon be testing the effects of these knockouts on tumor metastasis.

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